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Synthesis, Microbiological Activity and *In Silico* Investigation for Some Synthesized Coumarin Derivatives

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Abstract: Four 4-hydroxycoumarin derivatives were synthesized and the structure was confirmed by NMR spectroscopy and Mass spectrometry. Tested compounds have shown significant antimicrobial activity against *Bacillus subtilis* subsp. *spizizenii, Bacillus cereus*, *Staphylococcus aureus,* and *Staphylococcus epidermidis*, and the effect of more halogens on the benzene nucleus, as well as the combination of halogen and alkyl groups, on the antimicrobial activity, was investigated.
According to the docking study, these compounds can operate simultaneously on two enzymes, amylase and gyrase (1BAG and 1KZN), which are known to play an important role in bacterial life. Obtained docking study parameters for tested compounds showed an association with the *in vitro* results of the antimicrobial activity of these compounds. *In silico* tests of molecular properties of the tested compounds showed that the compounds met Lipinski's rule of five. In this paper, the ADME parameters of tested compounds were also calculated: Caco2 (*in vitro* Caco2 cell permeability), HIA (human intestinal absorption), MDCK (*in vitro* Mandin Darby Canine Kidney (MDCK) cell permeability), TPSA (topological polar surface area), etc.

Keywords: coumarins, docking, antibacterial activity, ADME.

INTRODUCTION

ONE of the reasons that drive researchers to synthesize new substances is the resistance of microbes to applied antibiotics. Since the last century, there has been a big interest in coumarin substances, particularly in the synthesis of their derivatives with antimicrobial activity.[1,2] Antibacterial drugs that target only one enzyme often lead to bacterial resistance due to single-mutation. Drugs that can operate in multiple locations within the same enzyme or simultaneously act on several enzymes that are important for the metabolism of essential microorganisms reduce the ability of bacteria to develop resistance.[3]

In a rational design of new biologically active compounds, various synthetic methods have been used to connect two or more biologically active molecules into a new structure with improved activity compared to the initial molecules. The presence of coumarin moiety has an important pharmacological and therapeutic role due to anti-inflammatory, anticoagulant, anticancer, antimicrobic, and antineurodegenerative properties, therefore the scientific interest in these compounds is enormous. Numerous coumarin derivatives with biological activity have been synthesized.[4–8] On the other side, cinnamic acids are a group of aromatic carboxylic acids (C_6–C_3) appearing naturally in the plant kingdom.[9] Cinnamic acids are formed in the biosynthetic pathway leading to

phenylpropanoids, coumarins, lignans, isoflavonoids, flavonoids, stilbenes, aurones, anthocyanins, spermidines, and tannins.[10] In the last ten years, the interest of researchers on the cinnamic acid moiety has notably increased. Several reviews and studies have appeared in the literature focusing on a different medicinal application of cinnamic-related molecules, for example, antimicrobial activity.[11,12] Some coumarin derivatives contain both components: coumarin and cinnamic.[13–15]

The inhibition of bacterial enzymes is a well-known mechanism by which drugs induce antibacterial activity. DNA gyrase is a bacterial protein from the topoisomerase family, which is involved in DNA transcription and replication processes. *Escherichia coli* DNA gyrase is a type of topoisomerase II that introduces negative supercoils by utilizing the free energy generated by ATP hydrolysis. This step is essential for DNA transcription and translation processes, so gyrase is a suitable target for antibacterial agents. Several classes of antibiotics have been used as inhibitors of gyrase activity for years. Some of them show high efficacy: quinolones (norfloxacin), coumarins (novobiocin and clorobiocin), and cyclothialidines.[16]

Quinolones interact with subunit A of DNA gyrase, whereas cyclothialidins and coumarins interact with the B subunit of this enzyme. Clorobiocin and novobiocin are the most important members of the coumarin family. These drugs are natural compounds that inhibit gyrase activity by competitively binding to the ATP binding site. The coumarin binding site completely overlaps with the ATP binding site as previously shown on the crystal structures of the 24 kDa gyrase domain inhibitory complexes. It has been shown that binding properties of coumarin ligands to the 24 kDa fragment are quite similar to those of the B subunit. Because both classes of antibiotics have limitations and side effects, the search for new gyrase inhibitors remains of great importance. Numerous synthetic coumarins with limited side effects have been designed. In recent years, there have been numerous attempts to generate small molecules that exhibit better or novobiocin-like inhibitory activity. The crystal structure of the clorobiocin-24 kDa gyrase complex was published and the coordinates were stored in a protein database (Protein Data Bank, PDB). Gyrase is present in prokaryotes and some eukaryotes, but not present in humans. This fact makes gyrase a good target for antibiotics.[17] Alpha-amylase from *Bacillus subtilis* complexed with maltopentose (PDB ID: 1BAG) has been described as a target in docking studies.[18] Activation of the alpha-amylase receptor from *Bacillus subtilis* building a complex with maltopentose (1BAG) is associated with antibacterial activity. Based on this, the 1BAG receptor was selected as the biological target for the docking of the synthesized compounds. A correlation between microbial activity *in vitro* and the binding energy results of the docking study has been established. It has been shown that blocking this receptor leads to antibacterial activity. Binding of a ligand to an active site that is composed of 18 amino acids provides the best answer.[19,20]

4-Hydroxycoumarin derivatives showed good microbiological activity and interesting physicochemical properties in our previous studies.[14,15,21] We proved that the presence of halogen on the benzene nucleus of these compounds increases the activity. In this paper, we have gone a step further. We wanted to examine the effect of more halogens on the benzene nucleus, as well as the combination of halogen and alkyl groups on microbiological activity. Also, this study aims were to evaluate the affinity of the binding of selected synthesized 4-hydroxycoumarin derivatives (which contain in their structure coumarin and cinnamic acid residues) to bacterial enzymes gyrase and amylase and to correlate obtained parameters *in silico* with obtained results of *in vitro* antimicrobial activity of compounds.

Docking does not answer all the questions which are important in the initial stages of testing for biologically active compounds, so data about cell membrane permeability are also valuable. Therefore, in this paper, the next parameters were also calculated: Caco2 (*in vitro* Caco2 cell permeability), HIA (human intestinal absorption), MDCK (*in vitro* Mandin Darby Canine Kidney (MDCK) cell permeability), TPSA (topological polar surface area), etc.

EXPERIMENTAL

Materials and Methods

TESTED COMPOUNDS

All derivatives used in the test were synthesized at the Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Sarajevo, according to the previously published method.[21] For four synthesized derivatives structure was confirmed by NMR spectroscopy and mass spectrometry.

DETERMINATION OF MELTING POINT

Melting points of tested compounds were determined by DSC (Differential Scanning Calorimetry) thermal analysis on the device Diamond DSC (Perkin Elmer).

NMR SPECTROSCOPY

The spectra were recorded on Bruker AV600 NMR spectrometer, operating frequencies for ^{1}H and ^{13}C NMR were 600.130 MHz and 150.903 MHz, respectively. Samples were dissolved in 0.6 mL of deuterated dimethyl sulfoxide (DMSO-d_6). The scale for ^{1}H is calibrated according to the signal shift of tetramethylsilane (TMS) and for ^{13}C according to the signal shift of DMSO-d_6.

MASS SPECTROMETRY

The analysis was performed on an Agilent 7890A GC apparatus equipped with 5975 C MSD. An HP-5MSI capillary column (Agilent Technologies, 0.25 mm diameter, 30 m long, 0.25 μm film thickness) was used. Samples were injected in split mode, in a volume of 1 μL. The carrier gas (He) flow rate was 2.1 mL/min at 60 °C (at constant pressure). The temperature of the column is programmed linearly in the range of 60–315 °C with a temperature increase of 25 °C min^{-1}. The mass spectra were obtained in electron ionization mode (EI) with ionic energy of 70 eV. The scanning range was *m* / *z* 33–550. The identification of the compounds is based on the interpretation of ionic fragmentation. The ion source temperature was 230 °C and the quadrupole temperature was 150 °C.

ANTIMICROBIAL ACTIVITY OF TESTED COMPOUNDS BY DIFFUSION METHOD

Antimicrobial activity was tested on standard strains of microorganisms:

- *Bacillus subtilis* subsp. *spizizenii* ATCC 6633
- *Bacillus cereus* ATCC 11778
- *Staphylococcus aureus* ATCC 6538P
- *Staphylococcus epidermidis* ATCC 12228.

Tablets used as reference standards:

- gentamicin 30 μg;
- erythromycin 15 μg.

THE DIFFUSION METHOD PROCEDURE

The European Pharmacopoeia method of diffusion is based on a comparison of the diameter of the zones of inhibition of the test sample solutions with the zones given by the standards. One mg of the compound was dissolved in 1 mL of DMSO. The solution thus prepared was used for further testing. Müller-Hinton agar and nutrients A, B, E, and F were used for the determination of antimicrobial activity by diffusion method.

Holes 6–9 mm in diameter are made in a solid nutrient medium. The holes were made using a stainless steel drill pipe 10 cm high and 6 mm ± 0.1 mm in diameter. Before use, the tubes were sterilized by heating to 150 °C and cooled. The drilled holes were filled with test compounds and a 100 μL blank (solvent), as well as tablets with standard compounds. Prepared plates were left at room temperature (or 4 °C) for 1 to 4 hours to diffuse the test sample into the inoculated medium.

Plates were then transferred to a thermostat, where bacterial strains were incubated for 18 hours at 37 °C. Upon completion of incubation, with an accuracy of 0.1 mm, the zones of inhibition formed by the action of the test compounds were read. The susceptibility of the microorganisms to the test compounds was measured by the width of the inhibition zone and compared to the inhibition zone of the chemotherapeutic agent that served as the standard. In the case of the insensitivity of the microorganism to the test compound, the microbes grow along the edge of the sample hole, so there is no zone of growth inhibition. The inhibition zones of the tested compounds were measured on a Readbiotic device. The measurement was done by placing a plate on the flat plate of the device and moving the part of the device representing the ruler to read the zones of inhibition in millimeters.

DOCKING STUDIES AND AFFINITY ASSESSMENT FOR THE RECEPTOR

AutoDockTools (ADT) was used to prepare, perform, and analyze docking simulations. The Lamarckian Genetic Algorithm (LGA) was applied to search for the most energy-efficient conformers. During docking simulations, a maximum of 100 conformers of each compound was analyzed. The structures of the tested compounds were optimized in Chem3D Ultra 9.0.1. program using the AM1 semi-empirical quantum-chemical method. Docking studies were performed using AutoDock 4.0. program. Discovery Studio Visualizer software was used to prepare receptors and ligands. PyMol 1.1 software was used to finally visualize the best conformation (test compound-receptor). The 3D coordinates of the crystallographic structure of the receptor were taken from the Brookhaven protein data bank (www.pdb.org) under the characteristic PDB code for each receptor. For docking studies for antibacterial activity, topoisomerase II DNA gyrase enzyme whose 3D coordinates are in the protein database (PDB ID: 1KZN) and alpha-amylase from *Bacillus subtilis* complexed with maltopentose (PDB ID: 1BAG) were used as receptors. Docking results are expressed through three specific parameters for each compound: inhibition constants (K_i), binding energies, and hydrogen bond formation.

The inhibition constant [K_i] is a parameter that determines what concentration of a compound is required to reduce the maximum rate of an enzymatic reaction by half.[22] The lower the value of this parameter, the higher the inhibitory activity. The binding energy [ΔG] is a parameter inversely proportional to the stability of the ligand-receptor complexes tested, and is calculated according to the equation:

$$\Delta G = \Delta H - T\Delta S$$

where ΔG is binding energy, ΔH is enthalpy contribution and $T\Delta S$ is entropy contribution.

When a ligand binds to a protein, the enthalpy decreases as a result of favoring intermolecular interactions and forming intermolecular bonds, while entropy increases as a result of the loss of the number of degrees of freedom.[23]

MOLECULAR PROPERTIES OF TESTED COMPOUNDS

Log*P* (octanol/water partition coefficient) which is used to estimate lipophilicity is calculated by Molinspiration developed methodology as a sum of fragment-based contributions and correction factors (miLog*P*). Topological polar surface area (TPSA) is calculated from the surface areas that are occupied by oxygen and nitrogen atoms and by hydrogen atoms attached to them. Number of Rotatable Bonds (nRotb) as a measure of molecular flexibility, molecular weight (MW), number of hydrogen bond acceptors (nON), and hydrogen bond donors (nOHNH) were also calculated using online available software (www.molinspiration.com).

ADME PARAMETERS OF TESTED COMPOUNDS

Caco2 (*in vitro* Caco2 cell permeability), HIA (human intestinal absorption), MDCK (*in vitro* MDCK cell permeability), BBB (*in vivo* blood-brain barrier penetration), PPB (*in vitro* plasma protein binding) and SKIN (*in vitro* skin permeability) were calculated using online available software PreADMET (https://preadmet.bmdrc.kr).

RESULTS AND DISCUSSION

Chemistry

The structures of the compounds used in the *in silico* and *in vitro* tests are shown in Figure 1.

COMPOUND 1: 3-(3-(2,4,6-TRIMETHYLPHENYL)PROP-2-ENOYL)-4-HYDROXY-2*H*-BENZOPYRAN-2-ONE

Yield 66 %; m.p. 203.7 °C; ^{1}H NMR (DMSO-d$_6$) *d* / ppm: 2.25 (s, 3H, H-20), 2.38 (s, 6H, H-21/H-22), 6.94 (s, 2H, H-16/H-18), 7.34 (d, 1H, $J_{7,8}$ = 8.47 Hz, H-8), 7.40 (t, 1H, $J_{5,6}$ = 7.89 Hz, $J_{6,8}$ = 0.63 Hz, H-6), 7.78 (t, 1H, $J_{7,8}$ = 8.47 Hz, $J_{6,7}$ = 7.41 Hz, $J_{5,7}$ = 1.53 Hz, H-7), 7.92 (d, 1H, $J_{12,13}$ = 16.14 Hz, H-13), 8.03 (d, 1H, $J_{5,6}$ = 7.89 Hz, $J_{5,7}$ = 1.53 Hz, H-5), 8.16 (d, 1H, $J_{12,13}$ = 16.14 Hz, H-12); ^{13}C NMR (DMSO-d$_6$) *d* / ppm: 19.9 (C20), 20.1 (C21/C22), 100.3 (C3), 115.4 (C10), 116.2 (C8), 124.0 (C6), 124.8 (C5), 126.4 (C13), 128.9 (C16/C18), 130.0 (C15/C19), 135.8 (C7), 137.0 (C14), 138.7 (C17), 143.6 (C12), 153.8 (C9), 159.0 (C2), 179.7 (C4), 191.1 (C11); MS *m* / *z*: 334 (M$^+$, 5), 316 ((M-H$_2$O)$^+$, 100), 196 (62), 189 (13), 162 (12), 145 (18), 130 (28), 121 (47).

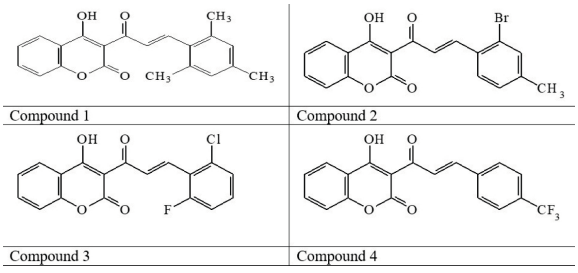

Figure 1. Structures of the tested coumarin compounds.

COMPOUND 2: 3-(3-(2-BROMO-4-METHYLPHENYL)PROP-2-ENOYL)-4-HYDROXY-2*H*-BENZOPYRAN-2-ONE

Yield 72 %; m.p. 209.2 °C; ^{1}H NMR (DMSO-d$_6$) *d* / ppm: 2.32 (s, 3H, H-20), 7.26 (d, 1H, *J* = 7.95 Hz, H-16), 7.32 (d, 1H, *J* = 8.43 Hz, H-8), 7.37 (t, 1H, *J* = 7.37 Hz, H-6), 7.51 (s, 1H, H-18), 7.67 (d, 1H, *J* = 7.95 Hz, H-15), 7.76 (t, 1H, *J* = 8.43, 7.37, 1.56 Hz, H-7), 7.98 (d, 1H, *J* = 7.89, 1.56 Hz, H-5), 8.16 (d, 2H, *J*=15,69 Hz, H-12/H-13); ^{13}C NMR (DMSO-d$_6$) *d* / ppm: 19.8 (C21), 100.5 (C3), 115.2 (C10), 116.1 (C8), 124.0 (C12), 124.0 (C6), 124.7 (C5), 124.9 (C19), 127.7 (C15), 128.6 (C16), 130.5 (C14), 133.1 (C18), 135.9 (C7), 142.6 (C13), 142.9 (C17), 153.7 (C9), 158.8 (C2), 179.5 (C4), 190.6 (C11); MS *m* / *z*: 386 (M$^+$, 4), 305 ((M-Br)$^+$, 100), 215 (8), 185 (17), 115 (19).

COMPOUND 3: 3-(3-(2-FLUORO-6-CHLOROPHENYL)PROP-2-ENOYL)-4-HYDROXY-2*H*-BENZOPYRAN- 2-ONE

Yield 62 %; m.p. 194.1 °C; ^{1}H NMR (DMSO-d$_6$) *d* / ppm: 7.31 (d, 1H, *J* = 8.58, 10.92 Hz, H-16), 7.36 (d, 1H, *J* = 8.40 Hz, H-8), 7.38–7.44 (m, 2H, H-6/H-18), 7.46–7.51 (m, 1H, $J_{C\text{-}17,\,F}$ = 9.6 Hz, H-17), 7.79 (t, 1H, J = 8.40, 1.41 Hz, H-7), 8.03 (d, 1H, *J* = 8.64, 1.41 Hz, H-5), 8.04 (d, 1H, *J* = 16.14 Hz, H-13), 8.45 (d, 1H, *J* = 16.14 Hz, H-12); ^{13}C NMR (DMSO-d$_6$) *d* / ppm: 100.8 (C3), 114.9 (C16), 115.1 (C10), 116.2 (C8), 124.1 (C6), 124.8 (C5), 125.8 (C18), 127.3 (d, $^3J_{C\text{-}14,F}$ = 15.4 Hz, C14), 129.4 (C12), 131.9 (d, $^2J_{C\text{-}17,F}$ = 9.6 Hz, C17), 133.9 (C19), 134.0 (C13), 136.0 (C7), 153.9 (C9), 159.0 (C2), 161.8 (d, $^4J_{C\text{-}15,F}$ = 249.0 Hz, C15), 179.3 (C4), 191.1 (C11); MS *m* / *z*: 326 ((M$^+$2-HF)$^+$, 33), 324 ((M-HF)$^+$, 100), 296 (49), 204 (50), 152 (100), 113 (52), 92 (48).

COMPOUND 4: 3-(3-(4-TRIFLUOROMETHYLPHENYL)PROP-2-ENOYL)-4-HYDROXY-2*H*-BENZOPYRAN-2-ONE

Yield 75 %; m.p. 180 °C; ^{1}H NMR (DMSO-d$_6$) *d* / ppm: 7.38 (d, 1H, $J_{7,8}$ = 8.32 Hz, H-8), 7.42 (t, 1H, $J_{5,6}$ = 7.93 Hz, $J_{6,8}$ = 0.87 Hz, H-6), 7.77–7.81 (m, 3H, H7/H16/H18), 7.92 (d, 2H, $J_{15,16}$ = 8.13 Hz, H15/19), 7.97 (d, $J_{12,13}$ = 15.84 Hz, H-13), 8.05 (d, 1H, $J_{5,6}$ = 7.93 Hz, $J_{5,7}$ = 1.62 Hz, H-5), 8.29 (d, 1H, $J_{12,13}$ = 15.84 Hz, H-12); ^{13}C NMR (DMSO-d$_6$) *d* / ppm: 100.2 (C3), 115.6 (C10), 116.9 (C8), 123.9 (q, $J_{C\text{-}18,F}$ = 272.7 Hz, C20), 124.8 (C6), 125.3 (C13), 125.4 (C12), 126.0 (C16/C18), 129.5 (C15/C18), 130.6 (q, $J_{C\text{-}17,F}$ = 30.9 Hz, C17), 136.8 (C7), 138.2 (C14), 143.7 (C5), 154.2 (C9), 159.5 (C2), 180.2 (C4), 191.6 (C11); MS *m* / *z*: 360 (M$^+$, 32), 239 (13), 215 (100), 199 (52), 171 (38), 151 (60), 121 (100), 92 (30).

Microbiology of Investigated Compounds

Results of the antimicrobial activity of compounds subjected to this test by diffusion method to Gram-positive aerobic bacteria (*Bacillus subtilis* subsp. *spizizenii, Bacillus cereus*, *Staphyloccocus aureus,* and *Staphylococcus epidermidis*) are shown in Table 1.

The values of inhibition zones of tested compounds ranged from 8 to 24 mm.

Three methyl groups of compound **1** contribute to the lipophilicity and good activity of this compound. Compounds having halogen chlorine and/or bromine, with zones of inhibition (ZI) of 16–23.75 mm, showed also a good activity. Compound **3** with fluorine and chlorine showed moderate activity. When the fluorine atom is directly attached to the benzene nucleus, due to its electronegativity it destabilizes coumarin molecule and by this probably causes poor activity. Unlike fluorine directly attached to the benzene nucleus (compound **3**), fluorine bound as a trifluoromethyl group significantly alters antibacterial activity. Thus, the greatest inhibition zone in the diffusion method was shown by the compound 3-(4-trifluoromethylphenyl)prop–2-enoyl)–4–hydroxy-2*H*–benzopyran–2-one (compound **4**), ZI = 24.00 mm toward *Bacillus cereus*. A similar activity was shown toward *Bacillus subtilis* (ZI = 23.00 mm) and slightly weaker toward *Staphylococcus epidermidis* (ZI = 20.00 mm) and *Staphylococcus aureus* (ZI = 19.00 mm). The trifluoromethyl group (CF_3) is lipophilic which contributes to the lipophilicity and activity of the whole compound.

Binding of 4-Hydroxycoumarins to Receptors

The basic interaction that allows ligand binding to the receptor is a hydrogen bond that is established between the polar groups of the amino acid branches in the peptide chain and the polar groups on the ligand molecule.

Compounds that can act in multiple sites within the same enzyme or simultaneously act on multiple enzymes important for the essential metabolism of the microorganism reduce the ability of bacteria to develop resistance. In this paper, the binding of 4-hydroxycoumarin derivatives to two enzymes was examined: alpha-amylase from *Bacillus subtilis* (PDB ID: 1BAG) and DNA gyrase subunit B from *Escherichia coli* (PDB ID: 1KZN).

Table 1. Values of inhibition zones (mm) of tested compounds against selected bacteria (by diffusion method).

Microorganism	*Staphylococcus aureus*	*Staphylococcus epidermidis*	*Bacillus subtilis*	*Bacillus cereus*
Compound		Zone / mm		
1	22.50	18.50	21.75	23.50
2	13.50	15.50	21.00	23.25
3	16.00	8.00	16.00	17.75
4	19.00	20.00	23.00	24.00
DMSO (control)	–	–	–	–
Erythromycin	24.20	29.00	32.00	23.00
Gentamicin	32.00	36.00	32.20	27.80

Binding of Compounds to the Alpha-Amylase from *Bacillus subtilis* (PDB ID: 1BAG)

The docking results of the selected coumarin derivatives on the 1BAG receptor are shown in Table 2.

Four 4-hydroxycoumarin derivatives were docked at the 1BAG receptor.

Compound **1** showed the binding energy of –5.17 kcal mol^{-1} and formed one hydrogen bond between the carboxyl group at position 2 of the coumarin ring and GLN-63.

Compound **2** showed the binding energy of –5.15 kcal mol^{-1} and formed one hydrogen bond. The carboxyl group at position 2 of the coumarin ring binds to the GLN-63 residue.

Compound **3** showed the binding energy of –4.63 kcal mol^{-1} and formed one hydrogen bond. The carboxyl group at position 2 of the coumarin ring binds to the residue of ASN-273.

Compound **4** exhibited the binding energy of –5.79 kcal mol^{-1} and builds two hydrogen bonds. Carboxyl group from the chain and the hydroxyl group at position 4 of the coumarin ring bind to the GLN-63 residue. This is the only compound in the series that builds two hydrogen bonds over the same amino acid.

As for structural analogs, the logical fact is that the amino acids of the proteins involved in the interaction are often the same. Thus, in most compounds, the amino acid residue of GLN-63 forms hydrogen bonds with the polar moieties of the compound molecules (compounds **1**, **2**, **4**). Polar parts of the molecules of the test compounds that build hydrogen bonds with the amino acid residues at docking with 1BAG are carboxyl group at position 2 of the coumarin ring, hydroxyl group at position 4 of the coumarin ring, oxygen from the coumarin nucleus at position 1 and carboxyl group from the chain.

Lower binding energies (the greater the negative number) indicate better binding of the compounds with bacterial amylase. Compounds with lower binding energies

Table 2. Docking results of coumarin derivatives at 1BAG: binding energies, inhibition constants, and amino acids involved in hydrogen bond formation.

Compound	1BAG		
	Binding energy / kcal mol^{-1}	Inhibitory constant / μmol dm^{-3}	Interacting amino acids
1	–5.17	162.58	GLN-63
2	–5.15	167.71	GLN-63
3	–4.63	403.22	ASN-273
4	–5.79	57.45	GLN-63
Chloramphenicol	–6.57	15.32	-

should also have lower inhibition constants to be considered as good enzyme inhibitors (as we can see in Table 2).

In docking analysis, chloramphenicol was used as the standard for interacting with 1BAG. The interaction of this drug with the receptor produced the lowest inhibition constant of 15.32 μmol dm^{-3}, while the binding energy was –6.57 kcal mol^{-1}.

Graphical simulations of binding of compounds **1**–**4** to receptor 1BAG are shown in Figure 2.

A comparison of the results obtained with docking with the results of antibacterial activity *in vitro* shows a relatively good concurrence.

Compound **4** (CF_3 group as a substituent) which showed the best *in vitro* activity on *Bacillus subtilis*, also showed almost the lowest binding energy (–5.79 kcal mol^{-1}) at 1BAG. This is the only compound in the series that builds over the same amino acid two hydrogen bonds. Also, this compound showed a low inhibition constant (57.45 μmol dm^{-3}). Compound **3** (F and Cl as substituents) showed the highest binding energy (–4.63 kcal mol^{-1}). This compound also had by far the highest inhibition constant of 403.22 μmol dm^{-3}. In *in vitro* studies, this compound exhibited poor antibacterial activity.

Binding of Compounds to the DNA Gyrase Subunit B from *Escherichia coli* (PDB ID: 1KZN)

The docking results of selected coumarin derivatives at the DNA gyrase subunit B are shown in Table 3.

Compound **1** exhibited a binding energy of –4.54 kcal mol^{-1} and builds two hydrogen bonds. Oxygen at position 1 of the coumarin ring binds to the residue of ASN-198, while hydrogen from the hydroxyl group at position 4 of the coumarin ring binds to residue SER-199.

Compound **2** showed a binding energy of –4.72 kcal mol^{-1} and builds one hydrogen bond. The Carboxyl group at position 2 of the coumarin ring binds to the residue of HIS-116.

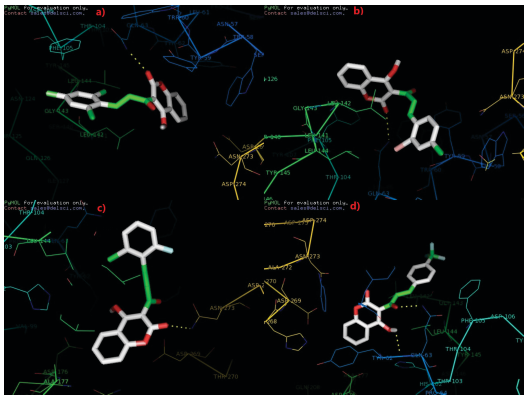

Figure 2. Binding mode of compounds **1** (a), **2** (b), **3** (c) and **4** (d) to receptor 1BAG.

Compound **3** showed a binding energy of –4.30 kcal mol^{-1} and does not form hydrogen bonds. Oxygen at position 1 of the coumarin ring forms hydrophobic interactions with the amino acid ASN-198.

Compound **4** showed a binding energy of –4.52 kcal mol^{-1}and builds one hydrogen bond. The carboxyl group in the chain binds to residue ASN-198.

As for structural analogs, the logical fact is that the amino acids of the proteins involved in the interaction are often the same. Thus, the amino acid residues of ASN-198 form hydrogen bonds with the polar moieties of the compounds **1** and **4**.

Polar moieties of the molecules of the test compounds that form hydrogen bonds with the residues of the amino acids of the DNA gyrase receptor are carboxyl group at position 2 of the coumarin ring, the hydroxyl group at position 4 of the coumarin ring, the oxygen from the coumarin nucleus at position 1, and the carboxyl group from the chain. Compounds with lower binding energies should also have lower inhibition constants (as we see in Table 3).

In docking analysis, clorobiocin was used as the standard for interacting with 1KZN. The interaction of this drug with the receptor resulted in an inhibition constant of 486.72 μmol dm^{-3}, while the binding energy was –4.52 kcal mol^{-1}.

Graphical simulations of the binding of compounds **1**–**4** to 1KZN receptor are shown in Figure 3.

The high binding energy was shown by compound **3** (F and Cl as substituents). This compound also had by far the highest inhibition constant of 704.12 μmol dm^{-3}. In *in vitro* studies, this compound showed poor antibacterial activity.

Predicting the Permeability of Derivatives Through Membranes

Unfortunately, docking, as a powerful and highly sophisticated method, does not answer the many questions that arise in the initial stages of testing for

Table 3. Docking results of coumarin derivatives at 1BAG: binding energies, inhibition constants, and amino acids involved in hydrogen bond formation.

Compound	1BAG		
	Binding energy / kcal mol^{-1}	Inhibitory constant / μmol dm^{-3}	Amino acids in hydrogen bonds
1	–4.54	471.77	ASN-198, SER-199
2	–4.72	345.92	HIS-116
3	–4.30	704.12	–
4	–4.52	483.12	ASN-198
Clorobiocin	–4.52	486.72	–

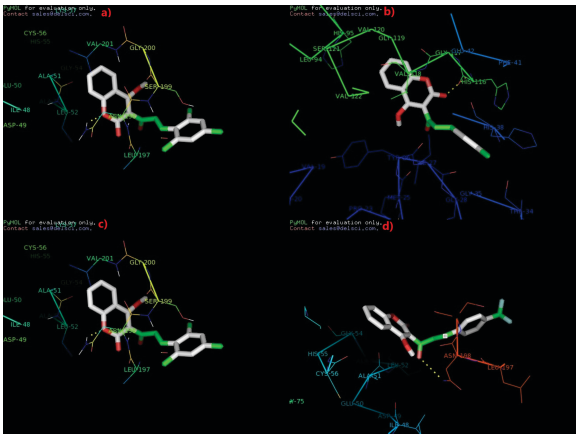

Figure 3. Binding mode of compounds **1** (a), **2** (b), **3** (c) and **4** (d) to receptor 1KZN.

biologically active compounds. Numerous other parameters provide information about the potency of some compounds to become drugs. The ability of the drug to pass through the membrane is one of the first parameters tested in the modeling of the new drugs. Lipinski's rule of five is well known.[24] According to Lipinski, a molecule will be able to pass the membrane by passive intestinal diffusion if it has: molecular weight of less than 500 g mol^{-1}, a log*P* value of less than 5 representing its hydrophobicity, no more than 5 hydrogen bond donors (HBD), and no more than 10 hydrogen bond acceptor (HBA) sites.[24] Further research has added two more parameters: topological polar surface area (TPSA) of less than or equal to 140 Å^2 and less than 10 rotatable bonds (Rotb),[25] which are correlated with drug permeability and flexibility.

In compliance with this set of rules, a chemical compound would act as an orally active drug-like compound on the desired target. If the molecule does not meet any two of the above requirements, it is assumed that it will not be capable of absorption by passive intestinal diffusion. From this rule, it is evident that the lipophilicity of the compounds is an important parameter in the process of intestinal drug absorption. But based on lipophilicity, not only the absorption but also the distribution, elimination, and toxicity of a drug can be predicted.

TPSA (Å^2) is calculated as a sum of fragment contributions (O- and N-centered polar fragments are considered). Since all four synthesized compounds have the same number of oxygen atoms located on the coumarin nucleus (have the same environment), the oxygen contribution is the same in all four synthesized compounds, and the TPSA value is identical.

The number of Rotatable Bonds (nRotb) is a measure of molecular flexibility. Rotatable bond is defined as any single non-ring bond, bounded to nonterminal heavy (i.e., non-hydrogen) atom.

Of the four synthesized compounds, compounds **1**, **2,** and **3** have the same number of rotatable bonds (3), while compound **4** has 4 rotatable bonds. The three common rotatable bonds are single bonds on a chain connecting the coumarin nucleus to the benzene ring while the fourth rotatable bond of the compound **4** is bond between the phenyl ring and the carbon of the CF_3 group. The results of permeability predicting for the tested compounds *via* passive intestinal diffusion, using Lipinski's rule of five, are shown in Table 4.

All tested compounds meet the Lipinski rule of five (Table 4), so it is assumed that they can be absorbed by passive intestinal diffusion.

ADME parameters of tested compounds are shown in Table 5.

Permeability through monolayers of human intestinal epithelial cells originated from human colorectal carcinoma cells (Caco-2) and Madin–Darby Canine Kidney cells (MDCK) are widely considered to be the *in vitro* gold standard for assessing the uptake efficiency of chemicals into the body.[26,27] Permeability through MDCK cell lines is also used to estimate the effect of the blood-brain barrier (BBB).[28–30] Given that these tests are time- and cost-intensive we calculated them using computer programs.

Table 4. Passage test of tested compounds *via* passive intestinal diffusion.

Com-pound	TPSA / Å^2	miLogP	MW / g mol^{-1}	nON	nOHNH	nRotb
1	67.51	4.55	334.37	4	1	3
2	67.51	4.53	385.21	4	1	3
3	67.51	4.09	344.73	4	1	3
4	67.51	4.42	360.29	4	1	4

TPSA - topological polar surface area; miLogP – LogP obtained by the Molinspiration; MW - molecular weight; nON - total number of hydrogen bond acceptors; nOHNH - total number of hydrogen bond donors; nRotb - number of rotatable bonds.

Table 5. Passage test of tested compounds *via* passive intestinal diffusion.

Compound	Absorption		PPB	BBB	SKIN	MDCK
	HIA / %	CaCo2				
1	95.98	16.86	91.86	0.34	–2.54	0.63
2	96.41	22.15	100.00	0.23	–2.60	0.04
3	96.08	22.29	100.00	0.14	–3.05	2.60
4	95.93	20.88	95.46	0.20	–1.95	0.05

Caco2 - *in vitro* Caco2 cell permeability (Human colorectal carcinoma) (nm/sec); HIA - Human intestinal absorption (HIA, %); BBB - *in vivo* blood-brain barrier penetration (conc.brain/conc.blood); MDCK - *in vitro* MDCK cell permeability (Mandin Darby Canine Kidney) (nm/sec); PPB – Plasma Protein Binding (PPB) - *in vitro* plasma protein binding (%); SKIN – *in vitro* skin permeability (transdermal delivery) (logK_p, cm hour^{-1})

Human intestinal absorption (HIA) is one of the most important ADME properties and also one of the key steps during the drugs' transporting to their targets.[31] "Poor" absorption was defined as HIA ≤ 30 %, "high" absorption as HIA ≥ 80 %, whereas "moderate" absorption was defined between these two values (30 % < HIA < 79 %). As can be seen from Table 5, high human intestinal absorption and *in vitro* plasma protein binding are predicted for all the synthesized compounds. The prediction of plasma protein binding (PPB) is of paramount importance in the pharmacokinetics characterization of drugs, as it causes significant changes in the volume of distribution, clearance, and drug half-life. The reversible interaction between drug and plasma protein can also greatly influence the pharmacological effect of the drug because only a fraction of unbound drug can pass across cell membranes. Thus, it can be expected that drugs with high protein binding tend to have a greater half-life compared to those with lower values. The greater the drug is bound to plasma protein, the less fraction of free drug is there for therapeutic effect.[32]

Determination of compounds' blood-brain barrier permeability is a prerequisite for screening compounds / bio-molecules which could take effects in the central nervous system.[33] As can be seen from Table 5, predicted *in vivo* blood-brain penetration for all the synthesized compounds is poor.

CONCLUSIONS

For the four synthesized 4-hydroxycoumarin derivatives, the structure was confirmed using NMR spectroscopy and Mass spectrometry. Microbiological studies have shown significant activity of these compounds. The *in silico* parameters obtained in the docking study of 4-hydroxycoumarin derivatives at 1BAG and 1KZN receptors show concurrence with the results of the antimicrobial activity of these compounds *in vitro*. According to docking, these compounds can operate simultaneously on two enzymes that are important for the metabolism of bacteria. In this way, the ability of bacteria to develop resistance to these compounds is reduced. Additionally, Lipinski's rule of five showed that all tested compounds can be absorbed by passive intestinal diffusion. Having in mind all the above, derivatives of this type are good candidates for further synthesis and research of the relationship between their structure and activity.

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